Identification and Validation of Novel Drug Targets for the Treatment of Plasmodium falciparum Malaria

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Chapter 12

Identification and Validation of Novel Drug Targets for the Treatment of *Plasmodium falciparum* Malaria: New Insights

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Abstract

In order to counter the malarial parasite's striking ability to rapidly develop drug resistance, a constant supply of novel antimalarial drugs and potential drug targets must be available. The so-called Harlow-Knapp effect, or "searching under the lamp post," in which scientists tend to further explore only the areas that are already well illuminated, significantly limits the availability of novel drugs and drug targets. This chapter summarizes the pool of electron transport chain (ETC) and carbon metabolism antimalarial targets that have been "under the lamp post" in recent years, as well as suggest a promising new avenue for the validation of novel drug targets. The interplay between the pathways crucial for the parasite, such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial tricarboxylic acid (TCA) cycle, is described in order to create a "road map" of novel antimalarial avenues.

Keywords: malaria, *Plasmodium falciparum*, drug design, drug target validation, protein interference, metabolic map, oligomerization

1. Introduction

"Portrait of a serial killer," a commentary published in 2002 in Nature Journal states: "Malaria may have killed half of all the people that ever lived" [1]. Despite the effort and funds spent on malaria eradication, it continues to infect approximately 200 million people worldwide every year and kill one in every four infected [2]. While effective in the past, current antimalarials are becoming less and less reliable as the parasite rapidly develops drug resistance [3]. There

have been a number of extensive reviews covering the recent status of antimalarial research and parasite's resistance [3–11]. The shared message highlighted in these articles is that a constant supply of novel antimalarials is urgently required. Similarly to the Harlow-Knapp effect described for human kinase research [12], the majority of the antimalarial research is currently aimed at optimization of existing drugs targeting the known and validated pathways.

The currently used antimalarial drugs can be classified into few classes based on the mode of action [3, 7]. Briefly, the groups that receive the most attention of the researchers include the artemisinins and chloroquine-like compounds, which target the food vacuole and heme processing and detoxification [13, 14], antifolates targeting the mitochondrial dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), such as proguanil [15, 16], and mitochondrial inhibitors targeting the electron transport chain and consequently the pyrimidine biosynthesis. Unfortunately, resistance has been reported for nearly all available treatments [3, 7]. Unsurprisingly, compounds such as artemisinin and quinolines that target a broad range of essential pathways within the parasite have successfully been used for nearly 40 years before the widespread of resistance had been reported. In contrast, single-target drugs, such as antifolates and atovaquone, have lost their efficacy within few years of clinical use [11, 17]. A number of promising approaches to counter the fast emerging drug resistance suggested by Verlinden et al. include extension of combination therapy to three or more orthogonal drugs, development and use of multitargeting compounds interfering with unrelated targets, and deeper look into the unexplored alternative targets [3]. In all three cases, in order to successfully overcome the parasite's remarkable ability to develop resistance to nearly all drugs used against it, by far, a number of novel validated drug targets must be significantly expanded.

This chapter summarizes the pool of the mitochondrial and carbon metabolism targets that have been "under the spotlight" in recent years, as well as suggest a promising new avenue for the validation of novel drug targets. We will focus on the interplay between the pathways crucial for the parasite, such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial TCA cycle, in order to create a "road map" for further antimalarial drug development.

2. The Harlow-Knapp effect

A scientific analogue of biblical "The rich get richer and the poor get poorer" can be rephrased as "the propensity of the biomedical and pharmaceutical research communities to focus their activities, as quantified by the number of publications and patents, on a small fraction of the proteome" [12] or the "Harlow-Knapp effect." It was first noted by Harlow and colleagues [18] and further expanded by Knapp group [19], based on the analysis of the amount of publications and patents featuring human protein kinases. Kinases are known to regulate the majority of the cellular pathways including those involved in cancer and other diseases. It was observed that despite the availability of human kinome [20] more than three quarters of protein research was still focused on just 10 per cent of the kinases that were already known before the kinome publication [21]. Edwards and co-workers have also noticed that "the availability of research tools influences a protein's popularity." In other words, scientists tend to further explore the well-known systems, ignoring the less studied biomolecules where the probing tools are yet unavailable.

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The availability of such tools for each system greatly limits the research opportunities and the attention to said system. Antimalarial research is not an exception to Harlow-Knapp effect: a limited opportunity for genetic manipulation [22] and complex life cycle of the parasite makes novel drug target validation highly challenging. Similarly to the human kinase research scientists tend to "keep looking under the spot light" among the few already validated targets, such as mitochondrial bc1 complex in malaria (target of the widely used Atovaquone), trying to optimize the existing compounds. Since first mentioned in the literature, there have been published more than 40 articles featuring plasmodial bc1 complex [23] and to the date it remains one of the most cited plasmodial enzyme.

Dihydroorotate dehydrogenase from *Plasmodium falciparum* (*Pf*DHODH) is another clear example of the Harlow-Knapp effect in antimalarial research. Since first proposed as a potential drug target more than a decade ago [24] and first inhibitors reported few years later [25], the major part of the research effort was focused on the optimization of the initial scaffold. In addition to the recent achievements in *Pf*DHODH inhibitor discovery by Phillips et al. [26], orthogonal methods, such as fragment-based drug design and virtual screening, have already yielded a number of very potent chemical scaffolds for this enzyme [27].

This divergent approach should be further exploited for other targets in order to yield novel and more potent scaffolds and support the antimalarial research.

3. Combinational therapy

The compound artemisinin and its derivatives have long been considered the most active and potent antimalarials for their efficacy against nearly all parasite stages [9, 14]. Artemisinins are believed to cause alkylation of proteins and heme and lead to oxidative damage within the parasite as well as affect the heme-related detoxification, although the exact mode of action is still a subject of debate [9, 14, 28]. Artemisinin-based combination therapy (ACT) is still recommended by World Health Organisation (WHO) for the treatment of uncomplicated falciparum and non-falciparum malaria in nearly all areas [7]. ACT implies the use of the fast acting artemisinin component, responsible for the rapid parasitemia clearance, in combination with another long-acting drug partner to eliminate the remaining parasites and suppress the selection of artemisinin resistance [29]. Despite the recent widespread of artemisinin-resistant falciparum malaria in Southeast Asia [30], the proven efficacy of combination therapy suggests that there is a pressing need for greater variety of highly effective antimalarial compounds. Combination of two or more drugs with different mode of action and resistance mechanisms significantly lowers the chances of the parasites to develop resistance to such treatment [31]. Thus, the research focus should be extended from optimization of existing compounds to development of novel research tools in order to explore and dissect other potentially druggable pathways of the parasite and thus bypass of the Harlow-Knapp effect. As stated by Verlinden et al.: "History has clearly indicated that new antimalarials must be continually developed in the ensuing event of resistance development to the current antimalarial arsenal." The occurrence of drug resistance in malaria is significantly faster than the development of antimalarials [3]. Thus, a constant supply of novel unrelated antimalarial compounds with orthogonal modes of action is urgently required.

4. The mitochondria as drug target for *P. falciparum* malaria

Mitochondria are organelles that act as the power plants of the cell, as they produce energy for all cellular activities. There are several molecular and functional differences between the mitochondria of *Plasmodium* species and those from the host. It is also known that the plasmodial mitochondria play a critical and essential role in the parasite's life cycle [5, 32, 33]. Previous studies have suggested that oxidative phosphorylation is not an essential pathway for parasite's survival during blood stage [34, 35]. In this stage, the parasite depends mainly on glycolysis as an energy source [36–38]. The observed glucose consumption in *P. falciparum*-infected red blood cells (RBC) was 75- to100-fold higher than in uninfected RBC [39]. Extraordinary glucose uptake during the infection leads to hypoglycemia, which together with an increased production of lactate and resulting lactic acidosis, are the major causes of mortality during severe malaria [40]. Thus, it is generally believed that the role of mitochondria in the parasite is not oxidative phosphorylation but the maintenance of the inner mitochondrial potential. Currently, the chemotherapeutic Malarone, a combination of mitochondrial *bc1* complex inhibitor Atovaquone and the dihydrofolate reductase inhibitor Proguanil, collapses the inner mitochondrial potential and induces parasite's growth arrest, confirming the mitochondrial metabolism to be crucial for the viability of the parasite. The importance of mitochondria for *Plasmodium* development in asexual stage is reinforced by the validation of another component of mitochondrial electron transport chain (ETC), dihydroorotate dehydrogenase (DHODH), as drug target [41, 42].

5. Electron transport chain (ETC)

The plasmodial mitochondrial electron transport chain (ETC) is composed of non-proton motive quinone reductases, such as dihydroorotate dehydrogenase (DHODH), malate-quinone oxidoreductase (MQO), glycerol 3-phosphate dehydrogenase (G3PDH), type II NADH dehydrogenase (NDH2, Alternative Complex I), and succinate dehydrogenase (SDH, Complex II), and proton motive respiratory complexes, including bc1 complex (Complex III), cytochrome *c* oxidase (Complex IV), and ATP synthase (Complex V) (**Figure 1**). The ETC requires ubiquinone (coenzyme Q) and *cytochrome c1* that function as electron carriers between the complexes [33, 44–47]. The (possible) roles of the ETC enzymes and their known inhibitors will be discussed in the following topics.

5.1. Dihydroorotate dehydrogenase (DHODH)

The *P. falciparum* enzyme dihydroorotate dehydrogenase (*Pf*DHODH) bridges the ETC and the pyrimidine biosynthesis; *Pf*DHODH catalyzes the key step of oxidation of dihydroorotate to orotate (a precursor for the biosynthesis of pyrimidine bases). The flavin mononucleotide (FMN)-dependent oxidation reaction catalyzed by DHODH can be divided in two half reactions: firstly, the oxidation of dihydroorotate through reduction of FMN and, secondly, the reoxidation of FMNH2 to regenerate the active enzyme. Two electrons resulting from this oxidation reaction are fed into the ETC through Flavin mononucleotide cofactor to

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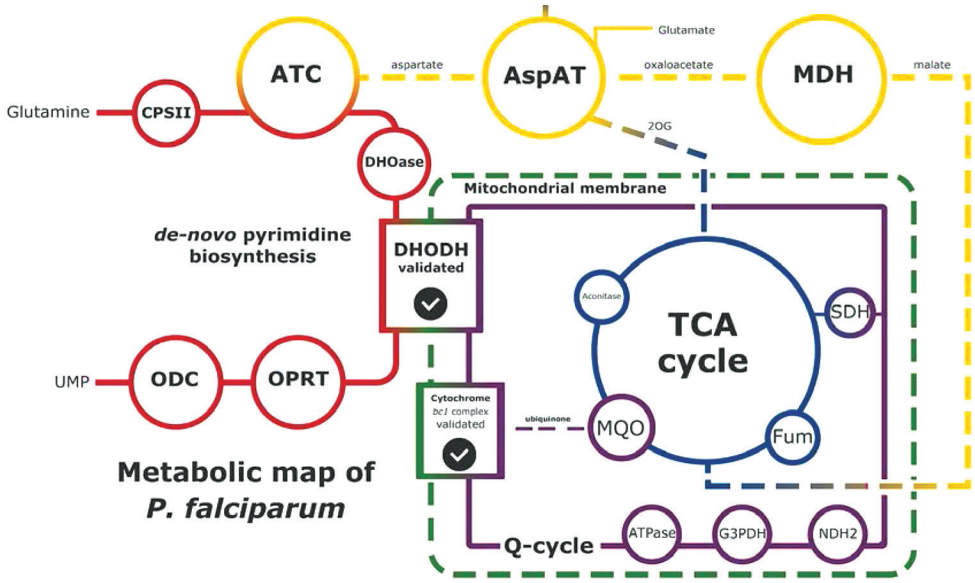

Figure 1. Suggested "roadmap" of essential metabolic processes of *Plasmodium falciparum* such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial TCA cycle. The map includes already-validated drug targets *Pf*DHODH [24] and cytochrome *bc1* complex [23, 43], as well as other promising targets.

ubiquinone, generated at the cytochrome bc1 complex, bridging pyrimidine metabolism and ETC [24, 48, 49]. Inhibition of *Pf*DHODH results in disruption of *de novo* biosynthesis of pyrimidines [48]. During the blood stage, the parasite depends strictly on this pathway for pyrimidine availability, which is essential for the formation of DNA, RNA, glycoproteins, and phospholipids [44].

Given the essential role of the *Pf*DHODH in the survivability of blood stage parasite and the significant differences to human DHODH [24], it is reasonable that the malarial enzyme has emerged as a novel validated drug target [26, 48, 50]. Inhibition of human DHODH was shown to be effective in treatment of autoimmune diseases, such as rheumatoid arthritis [51, 52]. The development of potent *h*DHODH inhibitors, such leflunomide and brequinar, led to the search of analogues with potential to inhibit plasmodial DHODH. These analogues were found to be poorly effective [53], potentially due to the differences in leflunomide and brequinar binding sites between human and plasmodial DHODH. These differences make *Pf*DHODH a potential species-specific drug target [24], which was extensively explored by a considerable number of studies. Although early research have not yielded effective results, the following studies have led to important achievements in the discovery of *Pf*DHODH inhibitors, such as benzimidazolyl thiophene-2-carboxamides [54–56], s-benzyltriazolopyrimidines [57], N-substituted salicylamides [58], trifluoromethyl phenyl butenamide derivatives [59], and triazolopyrimidine-based inhibitors [25, 60–64]. The triazolopyrimidine-based compound DSM265 was shown to be a potent inhibitor of the *Pf*DHODH and *Plasmodium vivax* DHODH

(*Pv*DHODH) with excellent selectivity versus *h*DHODH [48]. DSM265 has become the first DHODH inhibitor to enter the human antimalarial clinical trials, and preclinical development description was recently published, showing significant differences in DSM265 inhibitory activity between mammalian and plasmodial DHODHs. The kill rate of DSM265 for *in vitro* blood stage activity has shown to be similar to atovaquone, but significantly lower than observed for artemisinin and chloroquine. In addition, DSM265 has shown favorable pharmacokinetic properties, predicted to provide therapeutic concentrations for more than 8 days after a single oral dose in the range of 200–400 mg, what represents an advantage over current treatment options that are dosed daily. DSM265 was well tolerated in repeat dose, showed cardiovascular safety studies in mice and dogs, was not mutagenic, and was inactive against panels of human enzymes/receptors. Together, these data suggest that DSM265 has a high potential to be validated as a drug combination partner for either single-dose treatment or once-weekly chemoprevention [26].

5.2. Cytochrome bc1 (complex III)

The cytochrome bc1, also known as ubiquinol:cytochrome c oxidoreductase or complex III, is the only enzyme complex common to almost all respiratory ETCs [65]. This complex is composed of 11 different polypeptides, and its catalytic core is composed of three subunits, namely cytochrome b, cytochrome c1, and Rieske protein, also known as iron-sulfur protein (ISP) [66–68]. Cytochrome bc1 is found in the inner mitochondrial membrane and functions as a transporter of protons into the intermembrane space through the oxidation and reduction of ubiquinone in the Q cycle [67–70]. This enzymatic complex contains two distinct binding sites for the reduction and oxidation of ubiquinol and ubiquinone, both located within cytochrome b. The Qo site acts to oxidize ubiquinol near the intermembrane space, whereas the Qi site binds and reduces ubiquinone near the mitochondrial matrix [71, 72].

Although the crystal structure of plasmodial bc1 complex has not been solved, the high degree of sequence homology with other organisms of which the X-ray crystal structure is known (e.g. *Saccharomyces cerevisiae* [73]), allowed the discovery of many inhibitors. Cytochrome bc1 of *Plasmodium* is in fact a major drug target for the treatment and prevention of malaria and, to date, is the only component of the ETC with a clinically used antimalarial drug association [23, 43]. The compound atovaquone, a hydroxynaphthoquinone, inhibits cytochrome bc1 by binding to the Qo site. This inhibition leads to parasite death through the collapse of the *Plasmodium* mitochondrial membrane potential with no effect on the mammalian host [42, 74, 75]. Although atovaquone is a potent plasmodial bc1 complex inhibitor, its clinical utility is limited by the rapid emergence of resistant parasites when used as monotherapy [76]. Resistance to atovaquone has been developed due to mutations in the codon 268 (Y268S/C/N). These mutations affect the binding of the atovaquone to the target [77]. Because of that, atovaquone is used together with proguanil (Malarone) for treating uncomplicated malaria or as chemoprophylaxis for preventing malaria in travellers.

Aside of atovaquone, other bc1 complex inhibitors were described, as acridones [78], quinolones [79–81], pyridones [82, 83], and benzene sulfonamides [84]. Although many compounds have presented inhibitory potential against bc1 complex, this target might be considered

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underexploited, since the majority of these compounds target the Qo site [85]. The Qi site of cytochrome bc1 has been far less explored and only the binding of a few compounds has been reported [86–89].

5.3. Type II NADH dehydrogenase (NDH2)

Instead of the canonical multimeric complex I, or NADH:dehydrogenase, found in mammalian mitochondria, the *Plasmodium* ETC possesses the type II NADH:quinone oxidoreductase (NDH2). This enzyme, also known as alternative complex I, is a five quinone-dependent oxidoreductase enzyme involved in the redox reaction of NADH oxidation with subsequent quinol production [90]. Although the activity of NDH2 is still not biochemically confirmed in *P. falciparum*, it has been described in some detail for other organisms that also possess the type II NADH:quinone oxidoreductase, such as plants, fungi, and bacteria [91–96]. Differently from complex I, NDH2 is not involved in the direct pumping of protons across the membrane. Instead of proton pumping, NDH2 enables the H+-unregulated generation of mitochondrial reducing power supplying the various respiratory chains with reducing equivalents from NAD(P)H [45, 90].

So far, no crystal structure of the *P. falciparum* NDH2 (*Pf*NDH2) is available, and prediction of *Pf*NDH2 is based on sequence and structural similarities to other redox enzymes [45, 91, 97]. Although reverse genetics of *Pf*NDH2 was shown to be not lethal [98], *Pf*NDH2 was described as a putative "choke point" in the mitochondrial ETC and has been highlighted as a potential target for antimalarial development [45, 90, 99]. Given the lack of structural data for *Pf*NDH2 and its poor homology to any other structure in PDB, the existing studies aiming to inhibit *Pf*NDH2 for "druggable" proposes have used chemoinformatics and virtual screening methods. *Pf*NDH2 (as other NDH2 analogues) has shown to be insensitive to rotenone, a well-known inhibitor of complex I [90, 100]. The compound 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ), initially identified as an inhibitor of yeast NDH2 [101], was reported to be a potent inhibitor of *P. falciparum* proliferation [102]. In fact, HDQ inhibits *Pf*NDH2 but, in addition, it disrupts mitochondrial function through the potent inhibition of the bc1 complex [103]. The compounds dibenziodolium chloride (DPI) and diphenyliodonium chloride (IDP) have also been reported to inhibit *Pf*NDH2 activity in crude lysate fractions and both have shown efficacy against whole parasite proliferation [90]. However, a further study put the potential of *Pf*NDH2 inhibition by these compounds into question, since the authors were unable to corroborate the previous findings through dose-effect profiles using purified recombinant *Pf*NDH2 [100]. These results suggest that DPI and IDP may not be effective inhibitors of *Pf*NDH2, but their antiparasitic effect might be attributed to other enzymes instead (*e.g. Pf*DHODH) [100]. Inhibition of *Pf*NDH2 by artemisinin has also been demonstrated, suggesting a dual role for mitochondria in the action of artemisinin [104]. More recently, Antoine et al. [105] demonstrated that the low degree of inhibition of this enzyme by artemisinin indicates a non-ETC mode of action.

In more recent efforts, Biagini et al. [81] undertook a high-throughput screen (HTS) against *Pf*NDH2 using HDQ in combination with a range of chemoinformatics as starting point. This approach led to the selection of the quinolone core as the key target for SAR, followed by the selection of CK-2-68 as a lead for further development [81, 106]. Structural alterations aiming to improve the inhibitory activity and aqueous solubility led to the

compounds SL-2-64 and SL-2-25, the last presenting activity against *Pf*NDH2 and whole-cell *P. falciparum* at nanomolar range. *In vivo* experiments using *Plasmodium berghei*-infected mice demonstrated that SL-2-25 was able to clear parasitemia in the Peters' standard 4-day suppressive test when given orally a dose of 20 mg kg^{-1} [107]. SL-2-25, as other quinolones in this study, had the ability to inhibit both *Pf*NDH2 and cytochrome bc1 at low nanomolar range, the same dual inhibition previously observed for HDQ. This dual targeting of two key mitochondrial enzymes suggests that the quinolone pharmacophore is a privileged scaffold for inhibition of both drug targets.

Although the recent efforts to inhibit NDH2 with antimalarial purposes have been a good improvement in the knowledge of its potential as a drug target, the report of *Pf*NDH2 crystal structure would allow a deep investigation on both biochemical characterization and drug design targeting *Pf*NDH2.

5.4. Mitochondrial glycerol-3-phosphate dehydrogenase (mG3DH)

Mitochondrial glycerol 3-phosphate dehydrogenase (mG3DH) is a ubiquinone-linked flavoprotein embedded in the mitochondrial inner membrane that transfers reducing equivalents directly from glycerol 3-phosphate into the electron transport chain [108, 109]. The *P. falciparum* genome has homologues of both cytoplasmic and mitochondrial G3DH and assays indicate that the addition of glycerol-3-phosphate stimulates electron transport through the inner membrane [110–112]. Together with NDH2, mitochondrial G3DH from *P. falciparum* (*Pf*mG3DH) is also suggested to play an important role in the redox balance under conditions of low O_2. Further studies might clarify the essentiality of mG3PDH in *Plasmodium* survivability and also evaluate its potential as a drug target.

5.5. Succinate dehydrogenase (SDH)

The succinate dehydrogenase (SDH), also known as succinate: ubiquinone oxidoreductase (SQO) or complex II, is an enzymatic complex involved in both TCA cycle, functioning as a primary dehydrogenase, and in mitochondrial ETC, functioning as electron donator [113]. This dual role makes SDH a direct connection between major systems in aerobic energy metabolism. The enzyme has been isolated and characterized from prokaryotic [114–117] and eukaryotic organisms [118–121], including *P. falciparum* [122, 123]. SDH is located in the cytoplasmic membrane in bacteria [124] and in the mitochondrial inner membrane in eukaryotes [125]. The enzymatic complex is highly conserved and is basically composed of four subunits: a flavoprotein subunit (SDH1) and an iron-sulfur subunit (SDH2) together form a soluble heterodimer that binds to a membrane anchor b-type cytochrome (a CybL (SDH3)/CybS (SDH4) heterodimer). In *P. falciparum*, the two major subunits possess molecular masses of 55 kDa (Fp, flavoprotein subunit) and 35 kDa (Ip, iron-sulfur protein subunit) [122]. The SDH activity has shown to be essential for *Plasmodium* survivability, what makes this enzyme an attractive target for antimalarial development. The already reported differences in kinetic properties between *P. falciparum* SDH (*Pf*SDH) and human SDH increase the probability that *Pf*SDH inhibitors might represent potent and selective antimalarial compounds [122]. In fact, SDH has shown sensitivity to a number of inhibitors, such as 5-substituted 2,3-dimethoxy-6-phytyl-1,4-benzoquinone

derivatives, plumbagin and licochalcone [125], but so far, inhibitors with potential for antimalarial development still have to be discovered.

5.6. Malate-quinone oxyreductase (MQO)

The malate-quinone oxidoreductase (MQO) is a peripheral membrane-bound flavoprotein, which catalyzes the oxidation of malate to oxaloacetate, reducing ubiquinone [126]. *Plasmodium* species possesses a group 2 MQO, in contrast to bacterial group 1 MQO [127]. *P. falciparum* MQO (*Pf*MQO) is part of both mitochondrial ETC and TCA cycle, substituting other mitochondrial malate dehydrogenases (MDH) [111, 112, 128]. To date, no crystal structure of the *Plasmodium* MQO or inhibition studies are available. However, recent experiments showed that while knockout of six enzymes of plasmodial TCA cycle did not cause any significant growth inhibition, no viable MQO-knockout strains of *P. falciparum* could be obtained yet [34]. These findings as well as the absence of MQO in the human host make the enzyme an interesting target for antimalarial drug discovery.

5.7. ATPase

Although malaria parasites generate most of their ATP through aerobic glycolysis during the blood stage of their life cycle, they appear to possess a complete ATP synthase complex [47]. *P. falciparum* ATP synthase (*Pf*ATP synthase) is not reported to generate ATP but is suggested to act as a proton leak for the ETC [46, 47]. The use of bedaquiline, TMC207, has been proven to be effective for the treatment of multidrug-resistant tuberculosis. This compound targets *Mycobacterium tuberculosis* ETC through inhibition of ATP synthase rising the hypothesis that this may also be a valid drug target for malaria in the future [129]. So far, only one *Pf*ATP synthase inhibitor was described. The compound almitrine, originally developed as a respiratory stimulant, has activity against *Pf*ATP synthase and at the cellular level [130]. Recently, a genetic study demonstrated that mitochondrial ATP synthase is dispensable in blood stage *P. berghei*, although is essential in the mosquito phase [131]. For *P. falciparum*, previous attempts to knock out the mitochondrial ATP synthase subunits were unsuccessful, suggesting an essential role played by this enzyme complex in blood stages of the parasite [47]. The difference in essentiality of ATP synthase between *P. falciparum* and *P. berghei* could be explained by a possible distinction in the requirements of the two species for ATP [131]. Still, more studies are needed to define whether or not ATP synthase is essential in *P. falciparum* blood stage and consequently evaluate its potential as antimalarial target.

6. Tricarboxylic acid (TCA) cycle

While *Plasmodium* relies mainly on glycolysis during the blood stage, the TCA metabolism does occur in asexual *Plasmodium*, but at low turnover [35]. The exact function of the plasmodial TCA cycle is still a subject of debate, as it does not seem to function like a conventional TCA cycle. In 2010, a branched TCA pathway has been suggested for the parasite [132] but further retracted [133]. It was proposed that plasmodial TCA enzymes function not only in the classical but also in the reverse direction, generating either reductive or an oxidative pathway,

depending on the direction. Both pathways would result in the generation of malate, which is subsequently exported from the mitochondria, with α-ketoglutarate (2OG) being antiported to feed both the oxidative and reductive pathways [132]. Depending on the nutrient availability, *Plasmodium* species might not excrete malate as metabolic waste, utilizing it for metabolic purposes [134].

Further metabolomic studies suggest that *P. falciparum* utilizes conventional TCA cycle during both sexual and asexual blood stages [35]. Functional respiratory chain appears to be essential for the maintenance of inner mitochondrial membrane potential as well as protein and metabolite transport within the mitochondrion. The authors have also reported an increased sensitivity of gametocyte stages to sodium fluoroacetate (NaFAc). NaFAc was previously reported to inhibit TCA cycle enzyme aconitase in *Leishmania* [135]. Both sexual and asexual cultures of *P. falciparum* treated with 1 mM NaFAc showed significant citrate accumulation in the parasite as well as decrease in downstream TCA metabolites, suggesting the specific inhibition of aconitase of *P. falciparum*. However, no significant growth inhibition of the asexual parasites was observed, while gametocyte development was significantly reduced. These findings provide a potential for future transmission-blocking therapy.

Recently, Ke et al. [34] reported significant flexibility in TCA cycle metabolism of *P. falciparum*. The knockout experiments with all TCA cycle enzymes showed altered substrate fluxes between mitochondrial and cytosolic pools in nearly all cases. Out of eight enzymes of the TCA cycle, knockout of six enzymes of the TCA cycle showed no detectable growth defects. However, the authors were unable to disrupt the genes encoding fumarate hydratase and malate-quinone oxyreductase, suggesting potentially essential role of these two enzymes in asexual parasite development.

Although the fully functional TCA cycle appears to be dispensable for parasite survival in asexual blood stages [34], the interplay of some TCA enzymes with other essential pathways still represents an interesting target for antimalarial drug development. Below, we describe the role of three enzymes (aspartate aminotransferase, malate dehydrogenase, and fumarate hydratase) in *Plasmodium* metabolism and also their potential for antimalarial drug discovery. Other enzymes involved in this pathway (e.g., *Pf*SDH, *Pf*MQO) were previously described within the ETC section (see above).

6.1. Aspartate aminotransferase

The enzyme aspartate aminotransferase (AspAT) catalyzes the reversible reaction of L-aspartate and α-ketoglutarate into oxaloacetate and L-glutamate. The AspAT from *P. falciparum* (*Pf*AspAT) was placed into the Ia subfamily, being the most divergent member of this group. The crystal structure of *Pf*AspAT reveals an architecture similar to that previously determined in the *Escherichia coli* (1B4X14–17) [136–139], yeast cytosolic [140], pig heart cytosolic [141], and mitochondrial and cytosolic chicken [142–144] homologues. *Pf*AspAT is a homodimeric enzyme [145, 146], and each subunit consists of a large PLP (cofactor) binding domain, a smaller domain, that shifts the enzyme from "closed" to "open" form in order to provide substrate binding and N-terminal region that stabilizes the interaction between the two monomers into a dimer [142, 147, 148]. Two independent active sites are positioned near

the oligomeric interface and are formed by residues from both subunits [146]. The active site is highly conserved between available AspATs, making the design of species-specific inhibitors very challenging. However, it is known that the active site requires the formation of a homodimer, and analysis of AspAT has highlighted the N-terminal region as being highly divergent from other AspAT family members in both sequence and structure [145, 146]. Such a divergence may allow a more specific interference with the parasitic AspAT oligomeric surfaces, which offers a unique opportunity to generate highly specific interference with protein function *in vivo*. Such an approach will be further discussed in this chapter.

6.2. Malate dehydrogenase

The enzyme malate dehydrogenase (MDH) catalyzes the reversible NAD(P)+-dependent oxidation of oxaloacetate to malate. Like other members of the NAD+-dependent dehydrogenase family, the MDHs possess two functional domains, the catalytic domain and the NAD+-binding domain. Protozoan MDHs are differentiated into two subdivisions: mitochondrial and cytosolic MDHs, the first being part of the TCA cycle, providing oxaloacetate for the generation of citrate and NADH to fuel the mitochondrial electron-transport chain. The mitochondrial MDH is absent in *P. falciparum*, being replaced by *Pf*MQO (described in ETC section). The cytosolic MDH is present in *P. falciparum* (*Pf*MDH) acting as a supplier of metabolites, such as malate, to the mitochondria and might be responsible for the generation of reducing equivalents to feed the respiratory chain [149].

The crystal structure of *Pf*MDH has recently been solved [150]. Analysis of *Pf*MDH structure revealed a tetrameric assembly, although isoforms of the enzyme from other species have been reported to be present as either dimers or tetramers. Similar to *Pf*AspAT, the oligomeric nature of *Pf*MDH and the low degree of evolutionally conservation of the oligomeric interface residues provide an opportunity for a highly specific protein interference approach (described further).

6.3. Fumarate hydratase

Fumarate hydratase (FH) is an enzyme that catalyzes the reversible conversion of fumarate to malate. Although *P. falciparum* contains a fumarate hydratase homologue (*Pf*FH), it differs substantially from the "class II" type enzyme found in yeast and mammalian cells [151, 152]. Instead, the *Pf*FH resembles the iron-sulfur-containing "class I"-type enzymes found in some bacteria and archaea [153]. *Pf*FH was shown to be essential to the asexual stages of the parasite [34]. *Pf*FH was initially suggested to be located within the mitochondrion [153], however, this localization is yet not entirely clear.

Fumarate is a side product of the purine salvage pathway and acts as metabolic intermediate of the TCA cycle. As previously mentioned, *P. falciparum* does not export fumarate as metabolic waste but converts the metabolite to aspartate through malate and oxaloacetate. Besides, *P. falciparum*-infected erythrocytes and free parasites incorporate labeled fumarate into the nucleic acid and protein fractions [153]. Taken together, these data provide a biosynthetic function for fumarate hydratase and suggest that this enzyme could therefore be targeted for the development of antimalarial chemotherapeutics.

7. Pyrimidine biosynthetic pathway

A key-step for spreading of malaria parasites in the human host is the extensive and rapid replication of parasite DNA, which depends on the availability of essential metabolites, such as pyrimidines [154, 155]. In the *Plasmodium* species, besides the DNA, the pyrimidine nucleotide is also involved in the biosynthesis of RNA, phospholipids, and glycoproteins [155–157]. Sequencing studies have revealed that, in malaria parasites, the genes encoding for the pyrimidine biosynthetic pathway enzymes have been conserved, whereas those responsible for pyrimidines salvage have not [158]. It means that, while human cells are able to acquire pyrimidines either through *de novo* synthesis or by salvaging, the malaria parasites lack pyrimidine salvage enzymes and depend exclusively on the *de novo* pathway as source of pyrimidines for their survival [5, 33]. *De novo* synthesis from carbamoyl phosphate and aspartic acid follows basically the same steps found in the human host and in other eukaryotes: orotic acid is formed by dihydroorotase (DHOase) and DHODH. The orotic acid is so turned into orotidine 5′-monophosphate (OMP) by addition to 5′-phospo-D-ribosyl-α-1-pyrophosphate, a step carried out by orotate phosphoribosyltransferase (OPRT). OMP is subsequently decarboxylated to uridine 5′-monophosphate (UMP), the precursor of all other pyrimidine nucleotides and deoxynucleotides needed for nucleic acid synthesis [159]. Excepting for *Pf*DHODH, which is discussed in the ETC topic, the enzymes involved in *de novo* pyrimidine biosynthesis pathway that could potentially be exploited for the discovery of novel antimalarials as discussed below.

7.1. Carbamoyl phosphate synthetase II

Carbamoyl phosphate synthetase II (CPSII) is responsible for the first step of the *de novo* pyrimidine biosynthesis, catalyzing the formation of carbamoyl phosphate in the cytosol from bicarbonate, glutamine, and ATP [160]. Differently from the human CPSII, CPSII from *P. falciparum* (*Pf*CPSII) is a monofunctional protein [155]. *Pf*CPSII also differs from its mammalian homologue by the presence of two inserted sequences, located between junctions of the glutamine aminotransferase and synthetase domains [161]. Although the absence of structural information and activity inhibitors, the druggable potential of this enzyme has already been demonstrated by the potent growth inhibitory effect of a synthetic ribozyme with specificity for the *Pf*CPSII gene over *P. falciparum* cultures [162]. The same synthetic ribozyme has shown no toxicity to mammalian cells. Other mini ribozymes were further redesigned to improve cleavage activities and metabolic stabilities [163]. These results suggest that the discover of compounds capable to inhibit *Pf*CPSII in a specific way might be promising antimalarial candidates, since ribozyme approaches have a significant more challenging application due to target accessibility, stability, specificity, and delivery efficiency [164].

7.2. Aspartate transcarbamoylase (ATC)

Aspartate transcarbamoylase (ATC, EC 2.1.3.2) catalyzes the condensation of aspartate and carbamoyl phosphate to form N-carbamoyl-L-aspartate and inorganic phosphate. Previous

studies with human tumor tissues showed significantly elevated levels of ATC nearly in all samples [165]. In *P. falciparum*, ATC is also present as monofunctional protein, unlike its human homologue. Although a number of publications suggest ATC from *P. falciparum* to be a promising drug target [166–168], it has not been fully characterized and no inhibitors have yet been reported. Recently reported crystal structure of the truncated *Pf*ATC revealed high level of sequence conservation among homologous enzymes from other organisms, especially in the active site area [169].

7.3. Dihydroorotase

Similarly to CPSII, *P. falciparum* dihydroorotase (*Pf*DHOase) is a monofunctional protein and thus differs from the mammalian host, in which the 36.7 kDa enzyme is located on the central part of the 240 kDa CAD multifunctional protein [170]. This enzyme catalyzes the reversible cyclization of N-carbamoyl-L-aspartate (CA-asp) to L-dihydroorotate (L-DHO) [159]. Orotate and a series of 5-substituted derivatives were found to inhibit competitively the purified enzyme from *P. falciparum* culture. In mice infected with *P. berghei*, 5-fluoro orotate and 5-amino orotate at a dose of 25 μg/g body weight eliminated parasitemia after a 4-day treatment, an effect comparable to that of the same dose of chloroquine. The infected mice treated with 5-fluoro orotate at a lower dose of 2.5 μg/g had a 95% reduction in parasitemia [171]. The moderate inhibition of *Pf*DHOase by L-6-thiodihydroorotate (TDHO) in cultured parasites induced major accumulation of CP-asp and growth arrest, similar to atovaquone [172]. The analysis of physical, kinetic, and inhibitory properties of the recombinant *Pf*DHOase performed by Krungkrai et al. suggests that specific inhibitors may limit the pyrimidine nucleotide pool in the parasite, but have no significant adverse effect to human host [173]. Although the low amount of information about *Pf*DHOase does not allow to confirm it as a good candidate to antimalarial development, the report of its crystal structure and biochemical characterization could clarify whether this enzyme is essential or not to the parasite's survivability.

7.4. Orotate phosphoribosyl transferase and orotidine 5′-monophosphate decarboxylase

The last two steps of the pyrimidine biosynthesis in *P. falciparum* are catalyzed by a heteromeric complex that consists of two homodimers of *Pf*OPRT and *Pf*OPDC encoded by two separate genes [174, 175].

The enzyme orotate phosphoribosyl transferase (OPRT) catalyzes the formation of orotidine 5′-monophosphate (OMP) from α-D-phosphoribosyl pyrophosphate (PRPP) and orotate, the fifth step of the pyrimidine biosynthesis [155]. The OPRT inhibitors reported so far includes the compound 5′-Fluoroorotate, an alternative substrate for this enzyme that was shown to inhibit the *in vitro* growth of *P. falciparum* at nanomolar range [176, 177] and to clear parasitemia from *P. berghei*-infected mice [171]. This antimalarial activity is related to the inactivation of malarial thymidylate synthase by 5′-fluoro-2′-deoxy-UMP metabolite through covalent binding to methylene tetrahydrofolate at the active site. The compound pyrazofurin has also been described as a moderate inhibitor of *P. falciparum* OPRT (*Pf*OPRT), inhibiting its activity at micromolar range by blocking the maturation of trophozoites to

schizonts [176, 178]. Interestingly, pyrazofurin does not affect the OPRT activity in mammalian cells [179].

A recent study of the transition state analogues of *Pf*OPRT also showed that despite the tight binding *in vitro*, the synthetized compounds failed to inhibit the parasite culture growth *in vivo* [180–182]. No growth inhibition was observed at high compound concentrations up to 100 μM, suggesting poor compound accessibility *in vivo*.

Recently, the crystal structure of *Pf*OPRT has been reported, which shows a homodimeric assembly, where each of two active sites include amino acids from both chains [183]. Despite the high level of homology with human OPRT, the active site of *Pf*OPRT has few amino acids that differ from *Hs*OPRT. The authors suggest that these differences might lead to the design of selective substrate-like inhibitors in the future.

Orotidine 5′-monophosphate decarboxylase (OPDC) catalyzes the final step of *de novo* pyrimidine biosynthesis pathway, the decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP), with no need for the presence of a cofactor or metal ion [184]. Many inhibitors of plasmodial OPDC have been described so far, being the most promising inhibitor, the nucleotide 5′-monophosphate analogue xanthosine 5′-monophosphate (XMP) [185]. XMP acts as a competitive inhibitor with tighter binding than OMP. The *P. falciparum* OPDC inhibition by XMP is highly selective, having a 150-fold preference for the malarial enzyme compared to human OPDC. Other inhibitors include the 6-iodouridine 5′-monophosphate (6-iodo-UMP) [186], 6-azidouridine 5′-monophosphate (6-N3-UMP) [187], barbiturate 5′-monophosphate (BMP) [185], 6-N-methylamino uridine [187], and 6-N,N-dimethylamino uridine [187]. Although a considerable number of *Pf*ODC inhibitors have been described and a crystal structure of *Pf*OPDC is available [188], a deeper investigation is necessary to clarify *Pf*OPDC as validated drug target.

8. Protein interference assay (PIA) as drug validation tool

We have recently proposed a novel promising drug-target validation approach that relies on common feature of all biological systems—oligomerization [22]. Oligomerization is a self-assembly of two or more copies of one protein molecule (or different molecules) into one object. Recent analysis shows that majority (60%) of non-redundant protein structures available in the Protein Data Bank (PDB) represent dimerization or higher oligomerization order (Hashimoto *et al.*, 2011). In many cases, the biological activity of a protein complex is dependent on correct oligomeric order. Oligomerization may be required for a number of reasons, including the correct active site or cofactor binding site assembly on the oligomeric interface or allosteric regulation. Examples where dimerization is crucial for the formation of active sites on the oligomeric interface include previously mentioned aspartate aminotransferase (AspAT) [22], aspartate transcarbamoylase (ATC), and orotate phosphoribosyl transferase (*Pf*OPRT) [183] from *P. falciparum*. In addition, the physiological assembly of *Pf*OPRT/*Pf*OPDC heterotetramer was shown to be more effective compared to the

monofunctional enzymes [189]. A number of recent publications also suggest that the protein oligomerization to be a key driving force in evolution [190–194].

Another important aspect of oligomerization is remarkable selectivity and binding affinity. Large surface area of the intraoligomeric interfaces and evolutional diversity allow oligomeric partners selectively bind to each other with no cross-reactivity in the system. In majority of cases, purification of oligomeric proteins from both native and recombinant sources can be performed without any foreign protein incorporations in the assembly. Unlike the active sites and cofactor binding sites where evolutionary constraints restrict the sequence diversity to retain the function, oligomeric interfaces are significantly less conserved among homologous proteins [195, 196]. Thus, small molecule compounds reacting with the conserved active site of target enzyme of the parasite will likely interact with the host's homologous enzyme.

Direct interference with protein self-assembly would provide an opportunity for a highly selective modulation of protein activity or function both *in vitro* and *in vivo*.

9. Making (breaking) bad proteins

The recently proposed protein interference assay (PIA) [22] involves the utilization of structural knowledge (data) and mutagenic modification of one (or more) partner proteins in the assembly. These modifications may affect the binding site for a cofactor, catalytic activity, or disrupt the oligomeric interface of the target protein. Thus, recombinant and, most importantly, controlled co-expression of both wild type and its inactive (hyperactive) mutant would allow the formation of the complex with modified activity *in vitro*.

Previously mentioned homodimeric *Pf*OPRT, as part of the *Pf*OPRT/*Pf*ORDC heterotetramer, could also be a subject to PIA. The active sites of *Pf*OPRT were reported to contain the amino acids from both subunits, suggesting that introduction of the active site mutants with modified activity *in vivo* would also affect the native *Pf*OPRT. This assay would potentially bypass previously observed difficulties with poor inhibitor accessibility and aid in validation of the enzyme as antimalarial drug target.

Despite the obvious limitation of PIA approach to oligomeric proteins, this assay would still allow partial assessment of the system of interest, as many of the studied pathways are likely to involve at least one oligomeric assembly. We suggest that PIA would also allow re-evaluation of the previously studied promising targets where conventional validation approaches have failed.

10. Conclusion

In order to assess a gene's product role, one must possess a set of tools, such as genetic manipulations (e.g. knockout, silencing etc.), to modulate the target function *in vivo*. Sufficient specificity (with little or no cross-reactivity) is essential for correct interpretation of the data.

Although genetic manipulations have been proven to be highly effective in model and fully defined systems, less studied and complex systems remain highly challenging. In many pathogenic systems, including human malaria, conventional genetic manipulation techniques or small molecule inhibitor approaches do not always provide the desired efficacy [22]. In a number of human pathogens, multiple life cycle stages in different hosts and vectors make both *in vitro* and *in vivo* target characterization challenging to approach. A number of classic techniques such as silencing RNA [197, 198] have already been reported to be non-effective in certain cases [199–202].

In addition, the use of small molecule inhibitor approaches *in vivo* is associated with high costs and is often limited due to the variety of host-specific reasons that are difficult to predict, such as rapid metabolism, poor membrane transport, or localization. For example, while a number of compounds were reported to inhibit *Pf*OPRT activity *in vitro* as well as clear parasitemia in *P. berghei*-infected mice, *in vivo* trials with *P. falciparum* have failed [180]. Thus, potential drug targets may remain unexplored due to the inability to use the existing validation tool set.

Insufficient amount of effective target validation tools significantly limits the understanding of human pathogenic systems and hinders the rate of novel drug development. A constant supply of robust and effective techniques is needed in order to successfully dissect yet unexplored parasitic pathways, provide the basis for rational drug design, and counter-balance the ability of many human pathogens to rapidly develop drug resistance. We believe that protein interference assay (PIA) will enrich the currently available research toolset.

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